Global gene expression profiles for life stages of the deadly amphibian pathogen *Batrachochytrium dendrobatidis*

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Amphibians around the world are being threatened by an emerging pathogen, the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*). Despite intensive ecological study in the decade since *Bd* was discovered, little is known about the mechanism by which *Bd* kills frogs. Here, we compare patterns of global gene expression in controlled laboratory conditions for the two phases of the life cycle of *Bd*: the free-living zoospore and the substrate-embedded sporangia. We find zoospores to be transcriptionally less complex than sporangia. Several transcripts more abundant in zoospores provide clues about how this motile life stage interacts with its environment. Genes with higher levels of expression in sporangia provide new hypotheses about the molecular pathways involved in metabolic activity, flagellar function, and pathogenicity in *Bd*. We highlight expression patterns for a group of fungalysin metallopeptidase genes, a gene family thought to be involved in pathogenicity in another group of fungal pathogens that similarly cause cutaneous infection of vertebrates. Finally we discuss the challenges inherent in developing a molecular toolkit for chytrids, a basal fungal lineage separated by vast phylogenetic distance from other well characterized fungi.

amphibian population decline | chytrid | fungal pathogenicity | genomics

Amphibian populations around the world have been experiencing massive declines for several decades, with both local and global extinctions reported (1). Although these declines have been precipitated by a number of interacting factors (2), the recently discovered fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) (3) is responsible for many of the observed catastrophic die-offs. *Bd* has been documented in hundreds of amphibian species worldwide (4), has decimated many local frog populations (5, 6), and remains a devastating threat.

Bd infects keratinized amphibian skin, and there are distinct morphological and physiological changes observed in frogs suffering from *Bd*-induced chytridiomycosis (7, 8). However, because most studies of *Bd* in the decade since its discovery have focused on ecology and population genetics (9, 10), very little is known about the molecular and cellular mechanism underlying the lethality of *Bd*.

The challenges in studying the molecular biology of *Bd* are compounded by its position in the poorly characterized Chytridiomycota. Chytrids are basal fungi, separated by a vast phylogenetic distance from any well characterized relatives (11). Approximately 1.0 to 1.5 billion years of branch length lies between this pathogen and other fungi with fully sequenced genomes (12).

The complete genome of *Bd* has recently been sequenced (J.E.S., E.B.R., M.B.E., and Joint Genome Institute, unpublished data), enabling experimental genomics in this species for the first time. Genomic data have been used with some success to begin understanding the genetic basis of pathogenicity in other pathogens of vertebrates (14, 15). Given the speed at which *Bd* is decimating host populations, whole-genome assays promise a relatively rapid way to gain mechanistic insight into *Bd* disease processes.

Here, we initiate a functional genomics approach to understanding the molecular biology of *Bd* and conduct whole-genome expression assays (i.e., quantification of RNA abundance) to genetically characterize *Bd* life stages. The life cycle of *Bd* is divided into two broad categories: substrate-independent and substrate-dependent (Fig. 1). *Bd* zoospores are free-living, flagellated, and substrate-independent. Zoospores have a relatively short activity period and travel relatively short distances (16). However, in nature they are critical in initiating the infection of amphibian tissue. Zoospores in *Bd* exhibit chemotaxis (17), so they likely play an active role in finding appropriate substrates to colonize. Once a zoospore encysts, the substrate-dependent portion of the life stage begins. Germlings develop into zoosporangia, which produce additional zoospores. Mature zoospores are released from sporangia and can reinfect the same substrate or return to the surrounding aquatic environment (18). In nature, sporangia are of particular interest because they grow and reproduce in host tissue, and are responsible for increased pathogen loads because they release additional zoospores.

Although we are ultimately interested in the interaction between *Bd* and its amphibian hosts in natural systems, here we use controlled laboratory culturing conditions to (*i*) obtain sufficient genetic material for whole-genome assays, (*ii*) compare zoospores and sporangia under identical conditions, and (*iii*) describe genetic differences between *Bd* life stages in the absence of an amphibian host. These whole-genome data provide a necessary baseline for all future studies that endeavor to document host-specific or condition-specific patterns of *Bd* gene expression.

Results

We used the complete genomic sequence of *Bd* to generate a species-specific, whole-genome array. We then compared gene expression profiles for substrate-independent (i.e., zoospore) and substrate-dependent (i.e., sporangia) samples grown under standard laboratory conditions. Particularly for reporting zoospore results, we refer to "RNA abundance" rather than "gene expression" because zoospores may contained stored transcripts (as described later). Because *Bd* is phylogenetically distant from other fungi with well characterized genomes, determining the exact function of *Bd* genes is often difficult. Therefore, patterns of expression are generally more robustly described for functional classes of genes rather than for individual genes. Herein

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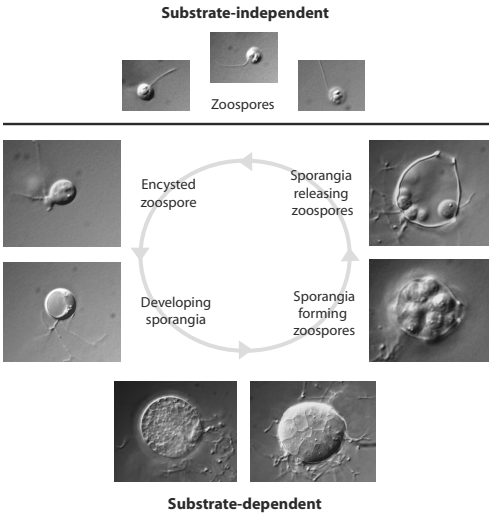

Fig. 1. The life cycle of *Bd*. In the substrate-independent portion of the life stage, flagellated zoospores are motile and free-living. In the substrate-dependent portion of the life cycle, zoospores encyst and develop into zoosporangia, which produce and release new zoospores.

we present results from two types of analyses: (i) analysis of enrichment patterns by using broad-scale functional classifications in the Gene Ontology (GO) database (19), and (ii) analysis of particular protein families and protein domains by using fine-scale functional classifications in the InterPro database (20).

Summary. The overall expression profiles of *Bd* life stages were strikingly different; more than half the genes in the genome exhibited differential expression between zoospore and sporangia samples. There are ≈9,000 genes in the *Bd* genome, 8,255 for which we designed probes. Of these 8,255 genes, 4,538 (55% of genes in the genome) satisfied our criteria of containing multiple statistically significant probes (at the $P < 0.05$ level after correction for multiple tests), all with differential expression in the same direction. In addition to differentially expressed genes, we recorded 1,522 "invariant" genes—those genes without any differentially expressed probes. Of the 4,538 genes with differential expression between life stages, 3,179 showed higher levels of expression in sporangia (39% of genes in the genome) and 1,358 showed increased RNA abundances in zoospores (16% of genes in the genome). Although we were able to functionally annotate the majority of genes in the *Bd* genome, it is important to note that a large number of *Bd* genes in our categories of interest currently have no GO or InterPro database numbers assigned (16% in the zoospore set, 14% in the sporangia set, and 28% in the invariant set). Additional genes of interest may therefore come to light as we learn more about the functions of currently unclassified genes.

Broad-Scale Patterns of Functional Enrichment. The GO database contains three different "ontologies" to describe the biological role of particular genes: biological process, cellular component, and molecular function. We searched for enrichment of functional categories in each of the three ontologies for each of the three gene categories of interest (i.e., higher expression in sporangia, increased abundance in zoospores, consistent representation in both samples). The GO database is organized as a network of nested functional categories, with "terminal nodes" being the most specific functional classification available. We define "unique terminal nodes" as those specific functional terms enriched in only one of our three gene lists.

Results for the molecular function ontology (Fig. 2) were generated by using our most stringently chosen gene set (≈100 genes per life-stage category). In this ontology, many terminal nodes were unique to each sample. Both the sporangia sample and the invariant sample showed enrichment for peptidase activity: metalloexopeptidases in the sporangia sample and serine peptidases in the invariant sample. If the less stringent gene set (≈1,000 genes per life-stage category) was used for the molecular function ontology, several additional unique nodes were observed: ligase and helicase activity in the zoospore sample and transmembrane transporter activity in the sporangia sample.

There was no consistent signature of enrichment for the biological process ontology by using our most stringently chosen gene set, however, many unique nodes were recovered by using our less stringent gene set (Fig. 2). In this ontology, genes with higher levels of expression in the sporangia sample were enriched for many aspects of carbohydrate and alcohol metabolism. Transcripts with greater abundance in the zoospore sample were enriched for phosphorylation and posttranslational protein modification. Invariant genes were involved in aspects of DNA metabolic process (e.g., DNA integration and modification), regulation of gene expression, and autophagy.

There was less resolution for the cellular component ontology for both the stringent and expanded gene sets. The only organelle with a life stage–specific enrichment pattern was the endoplasmic reticulum for the sporangia sample.

Fine Scale Patterns of Differential Expression. In addition to evaluating patterns of enrichment for broad GO functional categories, we also examined genes and gene families with specific InterPro functional domains of interest. There were more than

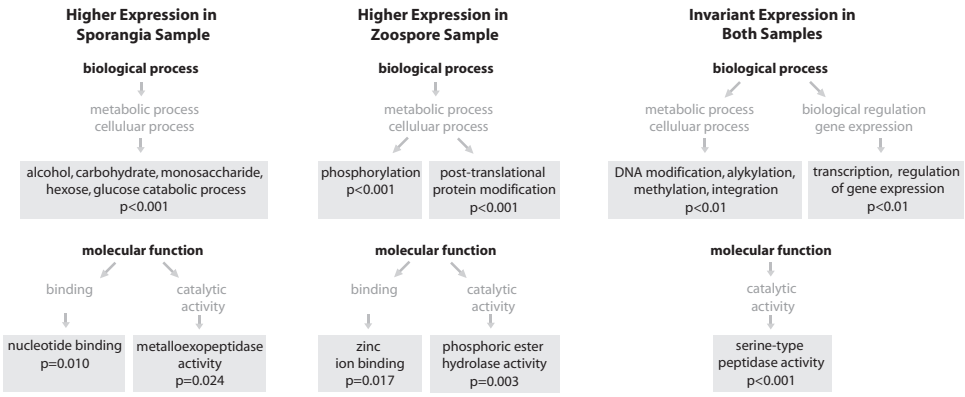

Fig. 2. Enrichment for GO terms in the biological process and molecular function ontologies. Gray boxes represent unique nodes for each sample.

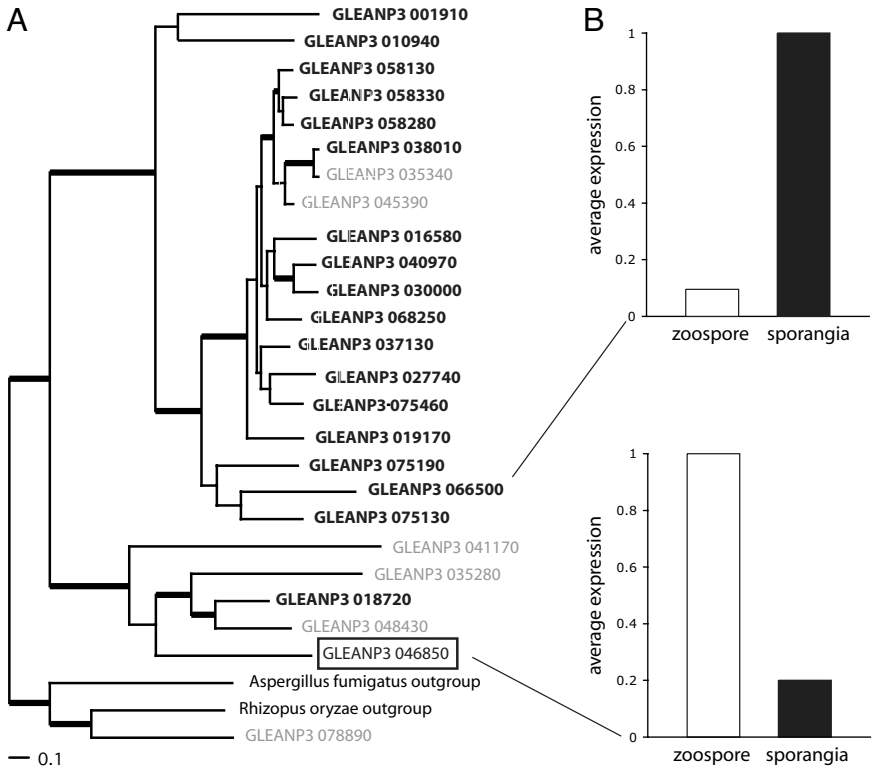

Fig. 3. Relationship and gene expression patterns for *Bd* fungalysin metallopeptidase genes. (*A*) Genes in bold font exhibited higher levels of expression in sporangia. RNA abundance for gene 046850 (boxed) was greater in zoospores. Gray gene names indicate *Bd* genes with no expression differences. (*B*) RT-PCR validation of patterns at genes 066500 and 046850 showing average relative expression for the two samples.

1,000 unique InterPro identifiers in our set of differentially expressed genes. Here, we restrict our discussion to those functional categories of particular relevance to *Bd* biology. Below we refer to specific *Bd* genes by number, which can be cross-referenced to the Joint Genome Institute's *Bd* portal (http://genome.jgi-psf.org/Batde5).

First we examined the expression profiles of putative pathogenicity genes. Several gene families have been hypothesized to play a role in pathogenicity in other disease-causing fungi, and we were particularly interested in candidate genes for pathogen interaction with vertebrate skin cells. We highlight expression data from two peptidase families, both inferred to have undergone expansions in the chytrid lineage (relative to the inferred common ancestor of all fungal species with full genomes available).

The fungalysin metallopeptidases (peptidase M36) may play a role in keratin degradation by dermatophytes, fungi that infect vertebrate skin (21). We observed a dramatic expansion of the fungalysin metallopeptidase gene family in *Bd*; there are 25 genes in the *Bd* genome with a peptidase M36 domain and probes on our array. Of these 25 genes, 19 (76%) show differential expression between life stages (Fig. 3). Nearly all of these (n = 18) exhibited higher levels of expression in the sporangia. However, there was one gene (046850) with a robust zoospore-specific pattern (31 of 40 probes had significant differences). By using RT-PCR, we confirmed patterns for this zoospore-specific fungalysin and one sporangia-specific fungalysin. With three biological replicates, we recorded fivefold higher levels of RNA abundance in the zoospore sample for gene 046850 and 10-fold higher levels of RNA abundance in the sporangia sample for gene 066500 (Fig. 3).

The serine-type peptidases (peptidase S41) also exhibit an expansion in *Bd* and similarly could be involved in host substrate or peptide degradation. Peptide degradation may be important for *Bd* because it is confronted with host antimicrobial peptides that are known to inhibit *Bd* growth (22). We found 29 genes in the serine peptidase family in the *Bd* genome, and of these, 41% showed differential expression between life stages. Most of these (n = 12) showed higher levels of expression in the sporangia sample (012080, 017900, 018730, 026250, 029020, 030810, 057050, 067480, 076390, 083780, 083870, and 085390), many with quite robust patterns (>30 probes per gene and >75% of probes giving a strong consistent signal). Only two peptidase S41 genes (022250 and 043530) displayed increased RNA abundances in the zoospore sample, and the patterns at these two genes were less robust (i.e., fewer probes with statistically significant differences in expression). An additional 41% of serine protease genes had invariant expression patterns.

Apart from genes with peptidase M36 or S41 domains, we did not identify other putative fungal pathogenicity factors in the *Bd* genome. However, there were several differentially expressed genes with sequence similarity to genes thought to be involved in viral or bacterial evasion of vertebrate immune systems [e.g., ITAM, interleukin-1 (002700, 026240)] (23, 24).

Second, we were interested in genes that could be involved in *Bd* flagella because flagellar function is critical to *Bd* zoospores. Flagella likely were present in the fungal-animal (opisthokont) ancestor, with subsequent losses of flagella on branches leading to the majority of extant fungal taxa (11). Therefore, we identified genes that are shared between *Bd* and Metazoa and are known to play a role in flagellar formation or function in other taxa. We identified two radial spoke genes (018230 and 063260) in the *Bd* genome with orthologs that are important in eukaryotic flagella; both showed higher expression in the sporangia sample. All four genes (028800, 039860, 060460, and 084220) with membrane occupation and recognition nexus (MORN) motifs, which are involved in a number of cellular processes, including

in animal flagella (25), exhibited higher levels of expression in the sporangia sample. In addition, several *Bd* genes with sequence similarity to genes implicated in mammalian sperm maturation and motility (e.g., Tcp11, Tpx-1) showed differential expression between life stages. However, these mammalian sperm function genes have fungal orthologs involved in different cellular processes, so it is premature to conclude a role for them in *Bd* flagellar function.

Third, a number of genes potentially involved in structural aspects of *Bd* cells displayed differential expression. Most notably, six of eight chitin synthases and three of six chitin binding genes showed differential expression between life stages, some with greater RNA abundances in zoospores (076580, 079190, 073240, 074430, 036850, and 052770) and some in sporangia (072590, 029580, and 088800). These genes each contained a large number of probes ($n = 22–66$), and patterns of differential expression were robust. Additionally, several genes involved in metazoan cell adhesion showed differential expression; two of two vinculin genes (014520 and 025140), two of two fibronectin genes (005350 and 039690), and one of four genes with a FAS1 domain (069890) exhibited higher expression in the sporangia sample.

Fourth, several genes involved in cell growth and cell death showed higher levels of expression in the sporangia sample. Three of seven cullin genes (029560, 069800, 081160), which are ubiquitin ligase subunits involved in controlling yeast cell division (26), and one TAP42-like protein (047320), which regulates a signaling pathway for cell growth in response to nutrients (27), showed higher expression in sporangia. The TAP42-like protein is the only gene in our set with a GO annotation of "response to biotic stimulus" (GO:0009607). Several apoptosis regulatory proteins also exhibited higher levels of expression in sporangia (031970 and 034360). Four of five universal stress protein genes (019030, 032770, 065040, and 079330) showed robust patterns of increased expression in the sporangia sample. Few genes displayed zoospore-specific patterns, but four of six genes in a particular class of cell division/GTP-binding proteins (010000, 023060, 036880, and 056890) had greater RNA abundances in the zoospore sample, with a large number of probes in each gene supporting this result.

Fifth, genes involved in cellular signaling and transport were of interest given the importance of the interaction of *Bd* with its environment. The G-α-protein genes, which play a role in cellular signaling (28), exhibit an expansion in *Bd*. In this family, transcripts from four genes were more abundant in sporangia (001760, 001770, 030330, 060140), three were more abundant in zoospores (001570, 001580, 020810). Most G-α genes with differential expression contained at least 30 probes per gene and had very robust patterns (up to 97% of probes with statistical significance). Additionally, two of two (001170 and 029610) peptidase A22 genes (presenilin) revealed zoospore-specific patterns. Although there are a large number of peptidase genes with aspartic catalytic activity in the *Bd* genome, the presenilins are thought to be important in signaling pathways (29). Last, in the *Bd* genome there are 22 clathrin- and clathrin adaptor–related genes (involved in cellular transport), and nearly 70% of them showed higher expression in the sporangia.

Discussion

Here, we provide a molecular profile of different life stages in the pathogenic chytrid fungus *Bd*. We compare global gene expression patterns for two portions of the *Bd* life cycle cultured under controlled laboratory conditions: the substrate-independent zoospore stage and the substrate-dependent sporangia stage. The two life stages exhibit strikingly different RNA abundance profiles, with 55% of genes in the genome showing differential expression. In the following paragraphs we discuss the development of a molecular toolkit for *Bd*, the genetic differences between *Bd* life stages, and some of the first hypotheses about pathogenicity factors in this species.

Gene Expression Differences Between *Bd* Life Stages. Global patterns of gene expression indicate that zoospores are transcriptionally less complex than sporangia, with RNAs from fewer than half as many genes showing increased abundances in the zoospore compared with the sporangia sample. Previous research in another chytrid, *Blastocladiella emersonii*, suggests that chytrid zoospores contain stored mRNAs and exhibit reduced RNA and protein synthesis (30). Our finding that genes involved in post-translational protein modification are enriched in zoospores is interesting in light of possible reduced transcriptional/translational activity in this life stage. Preliminary trials exposing *Bd* zoospores to the transcriptional inhibitor actinomycin-D did not compromise zoospore survival or motility and suggest that transcriptional provisioning may be adequate for basic zoospore function.

Because most genes in the *Bd* genome do not exhibit zoospore-specific patterns, genes with increased transcript representation in the zoospore sample are of particular interest because they provide a window into the pool of stored and newly transcribed mRNAs necessary for zoospore function. First, zoospores exhibit greater RNA abundances for several putative pathogenicity genes, a pattern discussed later. Second, we observed higher RNA abundances in the zoospore sample for several genes that may play a role in signaling. Several presenilin and G-α genes may provide clues for understanding how *Bd* zoospores interact with their environment. Third, we saw greater RNA abundances for several chitin synthases and chitin binding genes in the zoospore sample. Although chitin synthases with higher expression in the sporangia may be involved in cell wall remodeling during development, the function of these gene products in the zoospore remains to be determined. Finally, and perhaps surprisingly, we did not observe any putative flagellar genes with increased transcript representation in the zoospore sample. This is likely explained by the fact that zoospores develop within zoosporangia; all putative flagellar genes showed higher levels of expression in the sporangia sample.

In contrast to the zoospores, sporangia are transcriptionally and metabolically active and complex. The sporangia sample exhibited more than twice as many genes with higher levels of expression than the zoospore sample. The large number of genes involved in sporangia function is not surprising given that our substrate-dependent sample contained life-stage diversity not found in the zoospore sample—from encysted zoospores to mature sporangia. Our gene expression data capture this developmental variation and suggest some molecular mechanisms for the functional and structural complexity of sporangia. As expected, we saw higher levels of expression in our sporangia sample for genes involved in cell growth, cell death, and various aspects of alcohol and carbohydrate metabolism. A persistent gap in our knowledge of *Bd* biology is understanding what vertebrate cellular components are directly used by *Bd*. Although *Bd* attacks keratinized amphibian tissue, it is not clear that keratin is a primary nutrient source for this pathogen (16). Therefore, describing the metabolic pathways used by the substrate-dependent portion of the life cycle is important for gaining insight into the particular vertebrate cellular components necessary for *Bd* growth in host tissue. By identifying genes involved in sporangia metabolism, subsequent studies can evaluate the behavior of these genes under different nutrient and stress regimens.

Also of interest in the sporangia sample are a number of genes that have clear orthologs with metazoans. First, several genes (vinculin, fibronectin, fasciclin) involved in cell adhesion show higher levels of expression in the sporangia sample. Although *Bd* does not exhibit true multicellularity, sporangia can grow in a

colonial fashion and can appear in clusters. This "group effect," whereby *Bd* cells survive better in colonies, has been observed in laboratory conditions (3) and merits further dissection. Second, several genes involved in flagellar biogenesis in other taxa were identified in the sporangia sample. Radial spoke–like genes showed higher levels of expression in sporangia. These genes are well conserved in eukaryotes and play a role in flagellar movement of such diverse taxa as the green algae *Chlamydomonas reinhardtii* and the sea squirt *Ciona intestinalis* (31). Similarly, a number of genes with MORN motifs had higher levels of expression in sporangia. MORN motifs are found along many deep branches of the tree of life and have many hypothesized functions, including flagellar biogenesis in animal sperm. These motifs are found in proteins localized to sperm basal bodies and flagellum in the sea squirt *C. intestinalis* and the carp *Cyprinus carpio* (32). Zoospore development occurs entirely inside of zoosporangia, and flagellar genes expressed in developing sporangia are likely to provide more clues about the molecular basis for *Bd* flagellar development than pure zoospore samples. Synchronizing *Bd* cultures to examine expression profiles sliced more finely through the life cycle was outside the scope of this study but could provide additional insight into genes involved in zoospore development. Although it is difficult to obtain adequate amounts of RNA from synchronized cultures for whole-genome assays, it will now be feasible to analyze patterns throughout development at specific genetic targets identified here.

Gene Expression Patterns at Putative Pathogenicity Genes. We have identified two gene families with intriguing patterns of gene expression that are thought to specifically play a role in fungal pathogenicity. Serine protease and fungalysin metallopeptidase families both show large expansions in *Bd* and represent our most promising leads for understanding life stage–specific mechanisms of pathogenicity in *Bd*. Of the putative pathogenicity genes identified in this study, of greatest interest are the fungalysin metallopeptidase genes (peptidase M36). Not only did we observe an expansion of this gene family in *Bd*, but the vast majority (76%) exhibited differential expression between life stages. Nearly all differentially expressed fungalysins showed higher levels of expression in sporangia, the life stage associated in nature with keratinized host tissue. One fungalysin gene showed greater RNA abundance in the zoospore sample, and this gene may be an interesting target for understanding the initial stages of zoospore colonization of amphibian skin and entry into host cells. The serine protease family (peptidase S41) also requires further study in *Bd*; some genes in this family showed higher levels of expression in the sporangia sample, and others showed consistently high activity in both life stages.

Both fungalysin metallopeptidases and serine proteases have been implicated in pathogenesis in a variety of fungal pathogens that infect diverse animal hosts from nematodes to insects to humans (33, 34). Extracellular metallopeptidases are also expressed by a number of pathogenic fungi that specifically parasitize vertebrates (35). For example, multiplication of fungalysin metallopeptidases has been observed in dermatophytes, fungal pathogens causing cutaneous infections in vertebrates (21). *Bd* attacks keratinized amphibian tissue, and appears to exhibit chemotaxis toward soluble keratin in laboratory assays (17). However, it is not clear that *Bd* directly metabolizes keratin. Growth assays have suggested that *Bd* grows poorly and does not produce keratinases on pure industrial keratin (16), so it is important to consider the possibility that fungalysin metallopeptidases may be involved in degrading host cellular components other than keratin.

The methods used in the current study are not appropriate for conclusively demonstrating a functional role for particular peptidases in *Bd* pathogenicity or virulence. Rather, the purpose of our approach is to describe promising targets for further study. By identifying particular peptidase M36 and S41 genes with life stage–specific expression patterns in laboratory conditions, targeted follow-up studies can now be conducted to understand the role of these specific genes in the presence of host tissue.

In addition to factors thought to play a role specifically in fungal pathogenicity, several genes and gene families known to be involved in pathogenicity of more distantly related taxa showed differential expression between life stages in our study. We observed differential expression of a large number of clathrin-related genes, a gene family used by some pathogenic viruses and bacteria to enter host cells by mimicking ligands to the host's clathrin-dependent signaling system (36). Several genes that are involved in viral immune evasion by down-regulating host immune response also showed differential expression in our study. Although mechanisms of pathogenicity in viral, bacterial, protist, and fungal systems are likely to be quite divergent, motifs associated with ITAM, interleukin-1, and clathrin genes are conserved in vertebrates. It is possible that fungal pathogens could similarly mimic vertebrate host proteins to evade the immune system or make use of the host intracellular signaling machinery to gain entry into host cells. These possibilities are speculative and further work is necessary to determine whether the association of these genes with different *Bd* life stages is meaningful.

Genomics of Uncharacterized Fungi. The challenges of interpreting molecular profiles for uncharacterized organisms are augmented in *Bd* as a result of the difficulty in assigning orthology between *Bd* genes and those in phylogenetically distant taxa. Of genes lacking functional annotation, 58% displayed differential expression between life stages. Further, there is a large intersection between genes that appear to be unique to *Bd* and genes that showed increased RNA abundance in the zoospore sample. Understanding the molecular mechanisms that drive zoospore function in particular will likely require substantial new data acquisition for currently un-annotated genes in the chytrid genome. As we learn more about the genomes of basal fungi, our understanding of the functional classifications of *Bd* genes will become more refined and will provide additional insights into the molecular biology of this emerging pathogen.

Materials and Methods

Sample Preparation. Three *Bd* strains, isolated from different frog species, were used as biological replicates. These isolates were from a natural population of *Rana muscosa* from California (JAM81), a natural population of *Phyllomedusa lemur* from Panama (JEL423), and a captive population of *Xenopus tropicalis* from the University of California at Berkeley (JP005). Cultures were grown at room temperature on 1% tryptone, 1% agar plates for 2 weeks and then separated into two samples: (i) the substrate-independent sample contained free-swimming zoospores and (ii) the substrate-dependent sample contained all remaining *Bd* growth once zoospores were collected. To separate life stages, plates were flooded with water and incubated for 1 h so mature zoospores would release from zoosporangia. The water was then passed through a 20-μm Spectramesh filter (Spectrum Medical Instruments) to isolate zoospores. Additional water was added to plates and remaining growth was collected as the substrate-dependent sample. The two samples were pelleted and flash-frozen. It is important to note that the substrate-dependent sample was not homogenous: newly encysted, developing, mature, and empty sporangia were all captured in this sample. We designed our experiment to pool stages of sporangia growth because the comparison between free-living and substrate-embedded portions of the *Bd* life cycle is biologically relevant from a pathogenicity perspective. Additionally, our whole-genome approach required large quantities of RNA and precluded analyses of more finely sliced life stages.

Experimental Design. We designed a custom chip for *Bd* based on the complete genome of strain JAM81. The array contained 377,075 species-specific oligonucleotides, or $\approx$1 oligonucleotide every 100 base pairs throughout the genome. The two samples from each strain were labeled with different

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cyanine dyes and hybridized to a common chip to provide a direct comparison between life stages. Five chips were used for biological and technical replication. Samples for three chips were biological replicates (JAM81, JEL423, and JP005) and were prepared as a batch to ensure identical treatment. The remaining chips were technical replicates (one a direct comparison for JEL423 and one a dye-swap comparison for JAM81) and were treated individually to control for variation in technical conditions.

Molecular Methods. RNA was extracted with an RNeasy Mini Kit (Qiagen) with the standard protocol and a DNase digestion. Double-stranded cDNAs were synthesized by using the SuperScript cDNA Synthesis Kit (Invitrogen). cDNA samples were fluorescently labeled with custom Cy3- and Cy5-labeled oligonucleotides from TRILink BioTechnologies. A 16-hour hybridization was conducted at 42° in a Hybex microarray incubation system (SciGene). Chips were washed and then scanned on a GenePix Professional 4200 Scanner (Axon). We confirmed chip-based gene expression patterns for a subset of genes with RT-PCR by using SYBR green (Qiagen) and an ABI 7300 system (Applied Biosystems). We quantified gene expression of two target genes of interest relative to reference genes, which had high levels of expression in both the zoospore and sporangia samples.

Data Analysis. NimbleScan software (NimbleGen) was used to align a chip-specific grid to control features and extract probe-by-probe intensity data, and the LIMMA package in the R Project for Statistical Computing was used for statistical analysis. Global Loess normalization was used to normalize within arrays. Normalization among arrays was not necessary because mean and variance in intensities were similar across chips. A linear model was fit to the data, and a Bayesian *t* test was used to identify probes with significantly differential expression. The Benjamini and Hochberg method was used to control for the expected false discovery rate given multiple tests (37). Because there were multiple probes per gene, we used the following criteria to draw inference at the gene level. Genes were considered differentially expressed if (*i*) multiple probes in the gene showed significant differences in expression between life stages at the $P < 0.05$ level (after correction for multiple tests) and (*ii*) all probes in the gene with significant differences in expression showed higher expression in the same sample. In addition to identifying genes with differential expression between life stages, we identified invariant genes—those for which zero probes showed significant differences between zoospore and sporangia samples. We then conducted two related analyses. First, we looked for significant enrichment in GO terms by using GO Term Finder (38), a hyper-geometric test for enrichment at nested nodes throughout the GO ontology. Because so many genes showed differential expression, we modified our stringency criteria to reduce gene lists to ≈100 and ≈1,000 for each life stage for the GO analysis. For the sporangia sample, *P* value cutoff points of 0.0001 and 0.0005 gave us 126 and 745 genes, respectively. For the zoospore sample, *P* value cutoff values of 0.0005 and 0.005 gave us 81 and 810 genes, respectively. Second, we used InterPro identifiers to describe specific gene families and functional categories with differential expression between life stages. Finally, we identified several expansions in *Bd* gene families by evaluating the distribution of Pfam domains (39) across the currently available fungal genomes. Gene trees were constructed for the *Bd* expansions based on hidden Markov model–guided alignments to the Pfam domain and by using MrBayes (40) and RAxML (13).

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1. Loetters S, La Marca E, Stuart S, Gagliardo R, Veith M (2004) A new dimension of current biodiversity loss? *Herpetotropicos* 1:29–31.
2. Blaustein AR, Hatch AC, Beldon LK, Kiesecker J (2004) Multiple causes for declining amphibian populations. *Experimental approaches to conservation biology*, ed Gordon MS, Bartol SM (Univ of California, Berkeley), pp 35–65.
3. Longcore JE, Pessier AP, Nichols DK (1999) Batrachochytrium dendrobatidis gen et sp nov, a chytrid pathogenic to amphibians. *Mycologia* 91:219–227.
4. Skerratt LF, *et al.* (2007) Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth* 4:125–134.
5. Lips KR, *et al.* (2006) Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proc Natl Acad Sci USA* 3165–3170.
6. Rachowicz LJ, *et al.* (2006) Emerging infectious disease as a proximate cause of amphibian mass mortality. *Ecology* 87:1671–1683.
7. Parker JM, Mikaelian I, Hahn N, Diggs HE (2002) Clinical diagnosis and treatment of epidermal chytridiomycosis in African clawed frogs (*Xenopus tropicalis*). *Comp Med* 52:265–268.
8. Voyles J, *et al.* (2007) Electrolyte depletion and osmotic imbalance in amphibians with chytridiomycosis. *Dis Aquat Org* 77:113–118.
9. Morgan JAT, *et al.* (2007) Population genetics of the frog-killing fungus Batrachochytrium dendrobatidis. *Proc Natl Acad Sci* 104:13845–13850.
10. Morehouse EA, *et al.* (2003) Multilocus sequence typing suggests the chytrid pathogen of amphibians is a recently emerged clone. *Mol Ecol* 12:395–403.
11. James TY, *et al.* (2006) Reconstructing the early evolution of Fungi using a six-gene phylogeny. *Nature* 443:818–822.
12. Taylor JW, Berbee ML (2006) Dating divergences in the Fungal Tree of Life: Review and new analyses. *Mycologia* 98:838–849.
13. Stamatakis A (2006) RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22:2688–2690.
14. Dunning H, Julie C, Lin M, *et al.* (2006) Comparative genomics of emerging human ehrlichiosis agents. *PLoS Genet* 2:208–223.
15. Nierman WC, *et al.* (2005) Genomic sequence of the pathogenic and allergenic filamentous fungus Aspergillus fumigatus. *Nature* 438:1151–1156.
16. Piotrowski JS, Annis SL, Longcore JE (2004) Physiology of Batrachochytrium dendrobatidis, a chytrid pathogen of amphibians. *Mycologia* 96:9–15.
17. Moss AS, Reddy NS, Dortaj IM, San Francisco MJ (2008) Chemotaxis of the amphibian pathogen Batrachochytrium dendrobatidis and its response to a variety of attractants. *Mycologia* 100:1–5.
18. Berger L, Hyatt AD, Speare R, Longcore JE (2005) Life cycle stages of the amphibian chytrid Batrachchytrium dendrobatidis. *Dis Aquat Org* 68:51–63.
19. The Gene Ontology Consortium (2000) Gene ontology: Tool for the unification of biology. *Nat Genet* 25:25–29.
20. Mulder NJ, *et al.* (2007) New developments in the InterPro database. *Nucleic Acids Res* 35:D224–D228.
21. Jousson O, *et al.* (2004) Multiplication of an ancestral gene encoding secreted fungalysin preceded species differentiation in the dermatophytes Trichophyton and Microsporum. *Microbiology* 150:301–310.
22. Rollins-Smith LA, *et al.* (2006) Antimicrobial peptide defenses of the mountain yellow-legged frog (Rana muscosa). *Dev Comp Immunol* 30:831–842.
23. Smith GL, Chan YS (1991) 2 Vaccinia virus proteins structurally related to the interleukin-1 receptor and the immunoglobulin superfamily. *J Gen Virol* 72:511–518.
24. Geimonen E, *et al.* (2003) Hantavirus pulmonary syndrome-associated hantaviruses contain conserved and functional ITAM signaling elements. *J Virol* 77:1638–1643.
25. Inaba K (2007) Molecular basis of sperm flagellar axonemes: Structural and evolutionary aspects. *Reprod Biomech* 1101:506–526.
26. Michel JJ, McCarville JF, Xiong Y (2003) A role for *Saccharomyces cerevisiae* Cul8 ubiquitin ligase in proper anaphase progression. *J Biol Chem* 278:22828–22837.
27. Beck T, Hall MN (1999) The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. *Nature* 402:689–692.
28. Sandhya K, Vemuri MC (1997) Regulation of cellular signals by G-proteins. *J Biosci* 22:375–397.
29. Parks AL, Curtis D (2007) Presenilin diversifies its portfolio. *Trends Genet* 23:140–150.
30. Johnson SA, Lovett JS (1984) Gene expression during development of Blastocladiella emersonii. *Exp Mycol* 8:132–145.
31. Satouh Y, *et al.* (2005) Molecular characterization of radial spoke subcomplex containing radial spoke protein 3 and heat shock protein 40 in sperm flagella of the ascidian Ciona intestinalis. *Mol Biol Cell* 16:626–636.
32. Ju TK, Huang FL (2004) MSAP, the Meichroacidin homolog of carp (Cyprinus carpio), differs from the rodent counterpart in germline expression and involves flagellar differentiation. *Biol Reprod* 71:1419–1429.
33. Xu J, Baldwin D, Kindrachuk C, Hegedus DD (2006) Serine proteases and metalloproteases associated with pathogenesis but not host specificity in the Entomophthoralean fungus Zoophthora radicans. *Can J Microbiol* 52:550–559.
34. Huang XW, Zhao NH, Zhang KQ (2004) Extracellular enzymes serving as virulence factors in nematophagous fungi involved in infection of the host. *Res Microbiol* 155:811–816.
35. da Silva BA, Santos ALS, Barreto-Bergter E, Pinto MR (2006) Extracellular peptidase in the fungal pathogen Pseudallescheria boydii. *Curr Microbiol* 53:18–22.
36. Bonazzi M, Cossart P (2006) Bacterial entry into cells: A role for the endocytic machinery. *FEBS Lett* 580:2962–2967.
37. Benjamini Y, Hockberg Y (1995) Controlling the false discovery rate – a practical and powerful approach to multiple testing. *J R Statistics Soc Ser B* 57:289–300.
38. Boyle EI, *et al.* (2004) GO::TermFinder - open source software for accessing Gene Ontology information and finding significantly enriched Gene Ontology terms associated with a list of genes. *Bioinformatics* 20:3710–3715.
39. Finn RD, *et al.* (2006) Pfam: Clans, web tools and services. *Nucleic Acids Res* 34:D247–D251.
40. Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19:1572–1574.